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$GABA_A$ Receptors Mediate Trophic Effects of GABA on Embryonic Brainstem Monoamine Neurons *In Vitro*

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The inhibitory neurotransmitter GABA may act as a trophic signal for developing monoamine neurons in embryonic rat brain, because GABA neurons and their receptors appear in brainstem during generation of monoamine neurons. To test this hypothesis, we used dissociated cell cultures from embryonic day 14 rat brainstem, which contains developing serotonin (5-HT), noradrenaline (tyrosine hydroxylase; TH), and GABA neurons. Immunocytochemistry and reverse transcription-PCR (RT-PCR) revealed the presence of multiple α, β, γ, and δ subunits in these cultures. Competitive RT-PCR demonstrated high levels of β3 subunit transcripts. Expression of functional $GABA_A$ receptors was demonstrated using $^{36}Cl^-$ flux assays. To investigate GABAergic regulation of neuronal survival and growth, cultures were treated for 1–3 d *in vitro* with 10 μM GABA and/or $GABA_A$ antagonist (bicuculline or the pesticide dieldrin). The effects of treatments were quantified by analysis of immunoreactive 5-HT, TH, and GABA neurons. $GABA_A$ receptor ligands differentially regulated neuronal survival and growth depending on neurotransmitter phenotype. GABA exerted positive effects on monoamine neurons, which were countered by bicuculline (and dieldrin, 5-HT neurons only). By itself, bicuculline produced inhibitory effects on both 5-HT and TH neurons, whereas dieldrin potently inhibited 5-HT neurons only. GABA neurons responded positively to both antagonists, but more strongly to bicuculline. Taken together, these results demonstrate that the activation/inhibition of $GABA_A$ receptors produces opposite effects on the development of embryonic monoamine and GABA neurons. This suggests that these neurotransmitter phenotypes may express $GABA_A$ receptors that differ in fundamental ways, and these differences determine the developmental responses of these cells to GABAergic stimuli.

Key words: $GABA_A$ receptor; 5-HT; tyrosine hydroxylase; survival; neurite outgrowth; rat; bicuculline; organochlorine pesticides; dieldrin; RT-PCR; chloride influx; embryonic; brainstem

GABA is present in the mammalian brain during early stages of development, where it may act as a trophic signal for developing neurons. In the embryonic rat, GABA axons project through the brainstem when serotonergic (5-HT) and noradrenergic neurons are being generated (Lauder et al., 1986). This raises the possibility that GABA could exert trophic influences on developing monoamine neurons, if they express appropriate receptors. In adult rat brain, these neurons do express functional $GABA_A$ receptors (Smith and Gallager, 1987; Fritschy et al., 1992; Nicholson et al., 1992), but embryonic receptor expression has yet to be investigated. Trophic actions of GABA on other types of neurons are well documented (Spoerri and Wolff, 1981; Eins et al., 1983; Meier et al., 1984, 1991; Spoerri, 1988; Prasad and Barker, 1990; Hansen et al., 1991; Wolff et al., 1993; Abraham et al., 1994; Behar et al., 1994; Belhage et al., 1997) and seem to involve $GABA_A$ receptors coupled to Cl^- (Meier et al., 1985, 1991; Mehta and Ticku, 1988) and Ca^{2+} channels (Connor et al., 1987; Reichling et al., 1994; Obrietan and van den Pol, 1995; Xian et al., 1995) .

$GABA_A$ receptors are pentomeric complexes consisting of several subunits (α1–6, β1–4, γ1–3, δ, ρ1–2). Molecular cloning has identified a family of $GABA_A$ isoreceptors (subtypes) formed by several subunits from these classes (for review, see MacDonald and Olsen, 1994). These receptors are targeted by benzodiazepines, barbiturates, neurosteroids (Schofield et al., 1987; Barnard, 1988; Olsen and Tobin, 1990; for review, see Morrow, 1995), and organochlorine pesticides (Abalis et al., 1986; Gant et al., 1987; Costa, 1988; Bloomquist, 1992).

$GABA_A$ receptors develop in approximate spatiotemporal coincidence with GABAergic neurons and axons (Cobas et al., 1991; Schlumpf et al., 1989), suggesting that GABA may modulate expression of these receptors. *In situ* hybridization has revealed transient expression patterns of $GABA_A$ subunit transcripts in developing rat brain (Olsen and Tobin, 1990; Gambarana et al., 1991; Bovolin et al., 1992; Laurie et al., 1992; Poulter et al., 1992; Zheng et al., 1993; Ma and Barker, 1995). These subunits form functional $GABA_A$ receptors that can be activated by specific agonists (Hebebrand et al., 1988; Kellogg and Pleger, 1989; Fiszman et al., 1990; Schlumpf et al., 1992; Ma et al., 1993).

$GABA_A$ receptors have been suggested to mediate trophic effects of GABA during neuroembryogenesis (Ma and Barker, 1995). In the present study, we have used dissociated cell cultures from embryonic rat brainstem to study the effects of GABA on the growth and survival of developing monoamine and GABA neurons and have used $GABA_A$ antagonists to assess the involvement of $GABA_A$ receptors.

MATERIALS AND METHODS

Cell cultures

Primary dissociated cell cultures were prepared from embryonic day 14 (E14) rat brainstem as described previously (Liu and Lauder, 1991). Cells were plated in 12-well plates in complete medium [DMEM + 10% fetal calf serum (FCS) + penicillin/streptomycin/dextrose] at a density of 10^6

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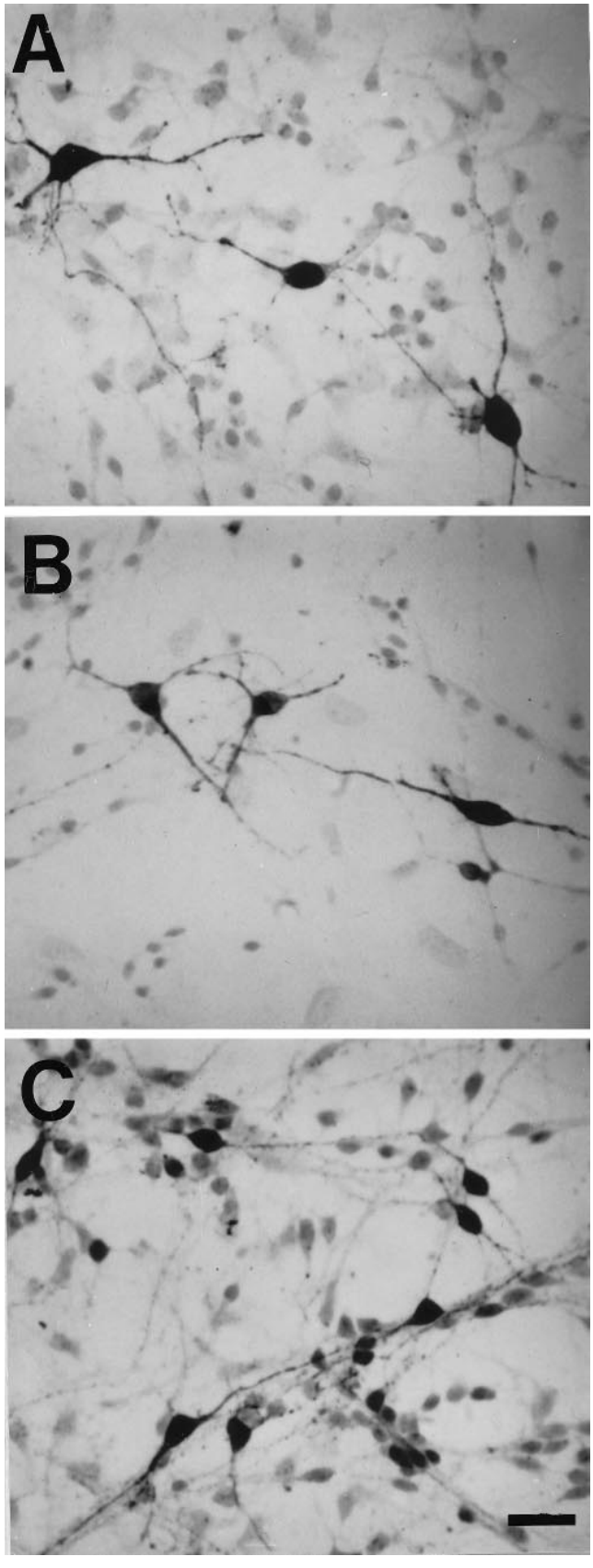

Figure 1. Representative 5-HT (*A*), TH (*B*), and GABA (*C*) immunoreactive neurons from E14 brainstem cultures. Cells were cultured for 1 d in DMEM + 10% FCS and then switched to serum-free medium (DMEM + ITS + 0.1% BSA) for 48 hr. Scale bar, 50 μM.

cells/milliliter on polylysine-coated coverslips. At 1 d *in vitro* (1 DIV), cultures were switched to serum-free medium [DMEM + insulin, transferrin, selenium (ITS) + 0.1% bovine serum albumin (BSA)]. To determine the effects on neuronal survival and growth, cultures were treated with 10 μM GABA and/or $GABA_A$ antagonist (bicuculline or dieldrin) in serum-free medium (to avoid GABA from serum-containing medium; Loscher, 1979) daily for 48 hr beginning at 1 DIV.

Immunocytochemistry and cell counts

At 3 DIV, cultures were rinsed with HBSS and fixed with 4% paraformaldehyde in 70 mM phosphate buffer. Cultures then were rinsed in PBS, permeabilized with 0.2% Triton X-100, and immunostained using the avidin–biotin peroxidase method (Vector Labs, Burlingame, CA) with specific rabbit polyclonal antisera against 5-HT-hemocyanin conjugates (Wallace et al., 1982), tyrosine hydroxylase (TH) (Boehringer Mannheim, Indianapolis, IN), GABA-hemocyanin conjugates (Lauder et al., 1986), $GABA_A$ receptor α1 subunits (Sato and Neale, 1989; gift of Dr. Joseph Neale), or a monoclonal antibody to $GABA_A$ receptor β2–3 subunits (bd-17, Boehringer Mannheim). To determine the effects of treatments on neuronal survival, the number of 5-HT, TH, and GABA immunoreactive neurons was counted in three cultures/treatment group using an ocular grid. Twenty grid areas (4.2 mm^2) were counted per culture. Data were expressed as the mean number of cells/mm^2/culture, and then converted to percentage control by dividing individual data points by the overall control mean. Data were statistically analyzed using one-way ANOVA followed by the *post hoc* Dunnet's multiple comparison test ($p < 0.05$).

Morphometry

Cell size, shape, and complexity of neurite outgrowth were measured for 5-HT, TH, and GABA neurons as indices of morphological changes in these neurons in response to treatments. Measured neurites consisted mainly of dendrites, because long, thin processes (presumably axons) were eliminated from the analysis. Morphometry was performed using a custom-designed computer imaging system, which automatically measured soma area (SA), field area (FA; area covered by a neurite arbor), number of neurites (NN), and number of terminal neurite segments (NTS), and then stored the images for further analysis. Details of this system and the morphometric parameters measured have been described previously (Lieth et al., 1990; Liu and Lauder, 1991; Lieth, 1992). In this experiment, 20 randomly selected neurons were analyzed from each of three cultures per treatment group. Data from these three experiments (total of 60 cells per group) were averaged to obtain means for each parameter ± SEM. Data ($n = 60$) were analyzed using one-way ANOVA followed by *post hoc* Bonferroni multiple comparisons.

Reverse transcription-polymerase chain reaction (RT-PCR)

RNA purification. Total RNA was extracted as described previously (Morrow et al., 1992). Culture media was removed from each well, and the cells were washed with ice-cold, sterile PBS. Prechilled 4 M guanidine thiocyanate was added to each of the wells, and then the solution from the combined six wells was transferred to centrifuge tubes, followed by homogenization on ice using a Polytron. RNA was purified by ultracentrifugation over a 5.7 M $CsCl_2$ cushion and resolubilized in 0.2 M sodium acetate (with 0.1% SDS). Extraneous protein was removed by consecutive extraction with equal volumes of Tris-buffered phenol and chloroform/isoamyl alcohol (49:1), followed by precipitation with 100% ethanol overnight. The yield of total RNA was determined by measuring the absorbance of an aliquot of the resuspended stock at 260/280 nm.

General RT-PCR. Aliquots of total RNA (1.0 μg) were reverse-transcribed in First Strand buffer (50 mM Tris, pH 8.3, 3 mM $MgCl_2$, 75 mM KCl) with 10 mM dithiothreitol, 1 mM deoxynucleotide triphosphates (dNTPs), 25 μM random hexamers, and 200 U Moloney Murine Leukemia Virus reverse transcriptase (BRL, Bethesda, MD) at 37°C for 60 min. The resulting cDNA was heat-denatured at 95°C for 10 min, and then tubes were put on ice until they were ready for PCR. The PCR reaction was conducted in PCR buffer (50 mM Tris, pH 9.0, 20 mM ammonium sulfate, 1.5 mM $MgCl_2$) with 50 μM each of 5′ (sense) and 3′ (antisense) primers, 200 μM dNTP, and 1 U Hot Tub Polymerase (Amersham, Arlington Heights, IL). The PCR reaction was performed with 26 cycles using a DNA Thermal Cycler, each cycle consisting of 94°C for 45 sec, 60°C for 45 sec, and 72°C for 1 min, followed by a final elongation step (72°C for 15 min). Aliquots of PCR products were run on a 1.8% agarose gel in 0.5 × TBE buffer.

Competitive RT-PCR reaction using internal standards. Competitive RT-PCR reactions using internal standards specific for α1, β3, and γ1 subunits

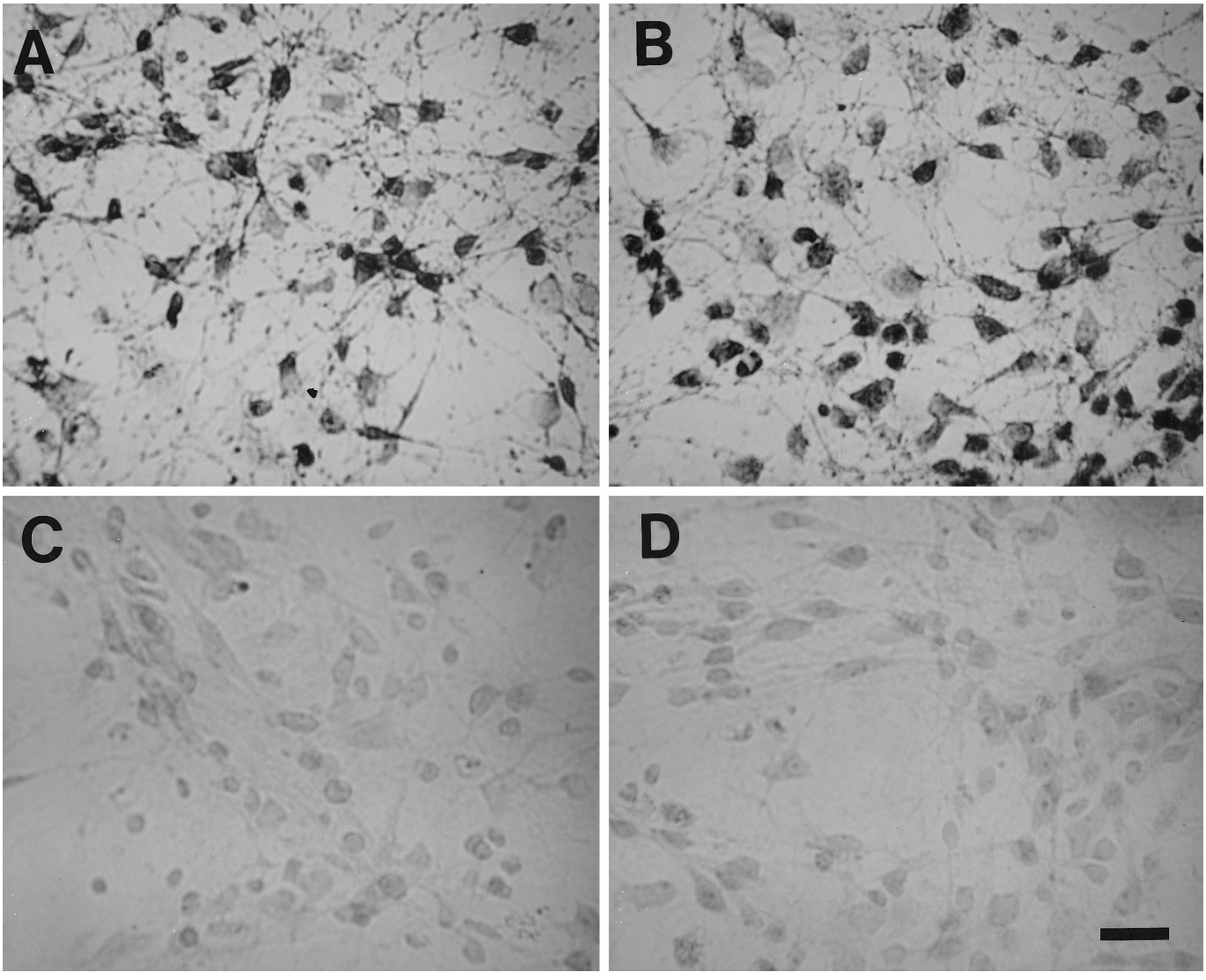

Figure 2. Expression of $GABA_A$ receptor subunit proteins in E14 brainstem cultures. Immunocytochemistry with anti-$GABA_A$ α1 rabbit polyclonal antibody (*A*) and anti-$GABA_A$ β2/3 monoclonal antibody (*B*). *C*, Background control with α1 primary antibody omitted. *D*, Background control with β2/3 primary antibody omitted. Cells were cultured for 1 d in DMEM + 10% FCS and then switched to serum-free medium (DMEM + ITS + 0.1% BSA) for 48 hr. Scale bar, 50 μM.

were conducted according to methods described previously (Bovolin et al., 1992; Grayson et al., 1993; Devaud et al., 1995). The cloned amplification products were sequenced to verify authenticity, and the primers were complementary to unique sequences within corresponding cDNAs (Bovolin et al., 1992). Various amounts of the internal standard cRNA (containing a *Bgl*II restriction site) were added to a constant amount of the total RNA of interest to generate a competitive PCR amplification curve. Each mixture was reverse-transcribed and run through PCR as in the general protocol, except for the addition of 1–2 μCi [^{32}P]dCTP/tube and the magnesium concentration, which was optimized for each primer pair and determined to be 1.5 mM for the α subunit mRNAs, 2.0 mM for β subunit mRNAs, and 3.0 mM for γ subunit mRNAs (Devaud et al., 1995). Aliquots of PCR products were digested overnight with *Bgl*II and separated on a 1.8% agarose gel. The gels were dried and exposed to a phosphorimaging screen for 1–2 hr. The signal intensity for both the native RNA products (~300 bp) and the cRNA products (~150 bp after digestion) was quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Data were presented as the ratio of amounts incorporated into the amplified cRNA internal standards to amounts incorporated into the corresponding subunit mRNA amplification product versus the known amounts of internal standard cRNA added to the test sample to generate a competitive PCR linear regression curve. The amount of target RNA was calculated directly from this curve (Grayson et al., 1993).

$GABA_A$ receptor-mediated $^{36}Cl^-$ influx into cultured cells

Chloride influx was measured in cultured brainstem cells by modification of a previously described method (Mehta and Ticku, 1988, 1992). Briefly, after culture of cells for 1 DIV in DMEM + 10% FCS followed by 2 DIV in DMEM + ITS + 0.1% BSA, coverslips were removed from culture wells and rinsed for 3–4 sec at room temperature in assay buffer (HEPES-buffered saline containing 20 mM HEPES, 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, and 2.5 mM $CaCl_2$, adjusted to pH 7.4 with Tris-base, with 10 mM glucose added just before use). Immediately after this rinse, the coverslips were drained rapidly on tissue paper and transferred to 3 ml HEPES-buffered saline containing 2 μCi $^{36}Cl^-$/ml (specific activity 13.38 Ci/gm), with or without various concentrations of GABA (5–60 μM) ± bicuculline (10 μM). Influx was terminated after 5 sec by transfer of the coverslip to 1000 ml ice-cold HEPES-buffered saline (with 100 mg/l picrotoxin) and then rinsed for 7 sec in another beaker containing 1000 ml ice-cold HEPES-buffered saline. Coverslips were then drained and transferred to scintillation vials containing 1 ml 0.2N NaOH. The vials were vortexed and then allowed to sit at room temperature for 1 hr. Aliquots (100 μl) were removed for protein determination using the Bradford method (Bradford, 1976). Retained radioactivity was determined by liquid scintillation spectroscopy. Values for $^{36}Cl^-$ influx were expressed as nanomol/milligram protein. Background levels

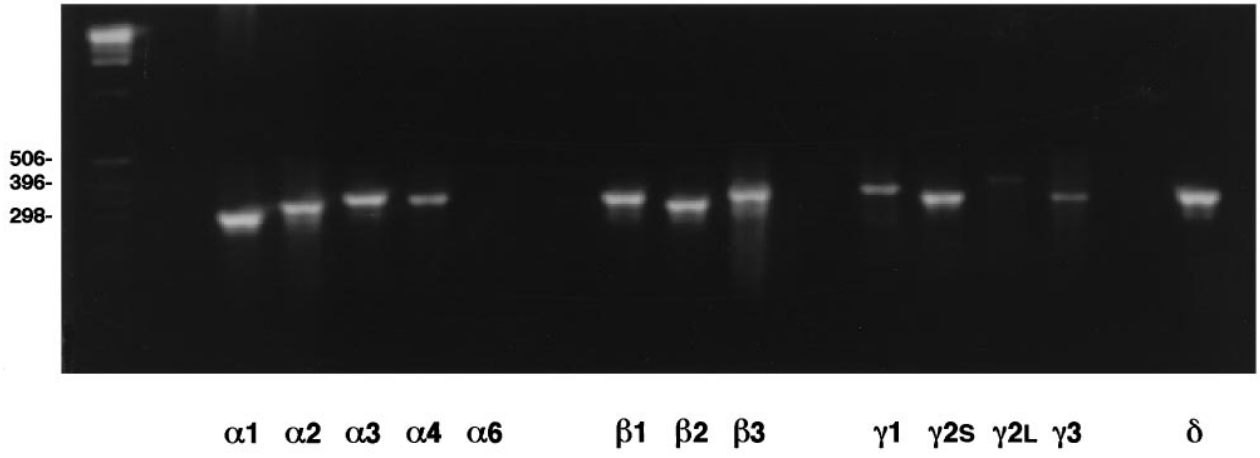

Figure 3. RT-PCR analysis of $GABA_A$ receptor subunit mRNAs in E14 rat brainstem cultures. Cells were cultured for 1 d in DMEM + 10% FCS and then for 2 d in serum-free medium (DMEM + ITS + 0.1% BSA). RT-PCR revealed expression of mRNAs encoding most of the known $GABA_A$ receptors, except α6.

of $^{36}Cl^-$ influx (without added GABA) were determined and subtracted from all values.

RESULTS

Embryonic brainstem cultures contain GABA and monoamine neurons, and express multiple $GABA_A$ receptor subunits

Immunocytochemistry demonstrated that 5-HT, TH, and GABA-immunoreactive neurons were present in E14 brainstem cultures (Fig. 1) and revealed extensive expression of α_1 and β2–3 $GABA_A$ subunit proteins, where dense immunoreactivity was localized to the outer membranes of neuronal somata and neurites (Fig. 2). General RT-PCR demonstrated the presence of mRNA transcripts encoding multiple α (α1–4, but not α6), β (β1–3), γ (γ1, γ2S,γ2L, γ3), and δ subunits of $GABA_A$ receptors (Fig. 3). Many of these subunits may be expressed by glial cells as well as by neurons in these cultures, because detectable levels of α1–3, β2–3, γ1, γ2S, and δ transcripts have also been found in purified glial cultures from E14 brainstem (J. Liu, R. Haberman, and J. Lauder, unpublished results). Quantitative competitive RT-PCR was performed for α1 and β3 subunits, because α1 and β2–3 antibodies had been used to characterize cultures immunocytochemically. The γ1 subunit was chosen for comparison. Competitive RT-PCR revealed that β3 subunit transcripts were highly expressed (272.5 ± 26.1 pg/μg total RNA) compared with α1 (1.9 ± 0.06 pg/μg total RNA) and γ1 (6.9 ± 0.3 pg/μg total RNA) transcripts (Fig. 4).

$GABA_A$ ligands regulate Cl^- influx, indicating the presence of functional $GABA_A$ receptors

GABA produced concentration-dependent and saturable effects on Cl^- influx in E14 brainstem cells at 3 DIV, with maximal enhancement at 10 μM GABA to ~13.6 nmol/mg protein (Fig. 5), which increased Cl^- influx over basal values by 400%. This effect was blocked by 10 μM bicuculline.

Neuronal survival and growth are differentially affected by $GABA_A$ ligands

Survival

The effects of GABA agonist and antagonist treatments (10 μM GABA and/or bicuculline or dieldrin in serum-free medium for 48 hr) on the density of 5-HT, TH, and GABA neurons are shown in Figure 6. GABA significantly stimulated survival of 5-HT and TH neurons but did not affect the survival of GABA neurons. The classical $GABA_A$ receptor antagonist bicuculline significantly reduced the survival of 5-HT and TH neurons, whereas the pesticide dieldrin reduced the survival of 5-HT neurons only. On the contrary, both $GABA_A$ antagonists significantly enhanced survival of GABA neurons. GABA completely blocked the effects of bicuculline on all three neuronal phenotypes, but did not reverse the effects of dieldrin on 5-HT and GABA neurons.

Growth

The effects of treatments (described above) on the growth of cell bodies and neurites are shown in Table 1.

SA: The *size of cell somas* (soma area) of TH neurons was significantly increased by GABA. When added together with GABA, bicuculline and dieldrin inhibited this growth effect but had no significant effect when given alone. Other treatments had no significant effects on cell somas of these neurons. Cell somas of GABA and 5-HT neurons were not affected significantly by any treatment.

FA: The *surface area covered by neurites* (field area) of 5-HT neurons was increased after treatment with GABA, whereas GABA had no significant effects on the FA of TH or GABA neurons. Dieldrin significantly reduced the FA in 5-HT neurons, an effect that was not overcome by co-administration of GABA, whereas bicuculline had no significant effect; however, bicuculline did decrease the FA in TH neurons, which was reversed by GABA. Although GABA itself had no significant effect on the FA of GABA neurons, bicuculline stimulated this parameter, which was blocked by co-administration of GABA. Dieldrin also seemed to increase the FA of these neurons to some extent, but this effect did not reach significance using the Bonferroni test (although it was significant by the less conservative *post hoc t* test).

NN: The *number of primary neurites* of TH neurons was increased significantly by GABA, which was reduced to nonsignificant levels by bicuculline. Although the one-way ANOVA for treatment effects on NN of 5-HT neurons was highly significant ($p < 0.001$), and *post hoc t* tests indicated significant growth-promoting effects of GABA and inhibitory effects of dieldrin, no treatment effect reached significance

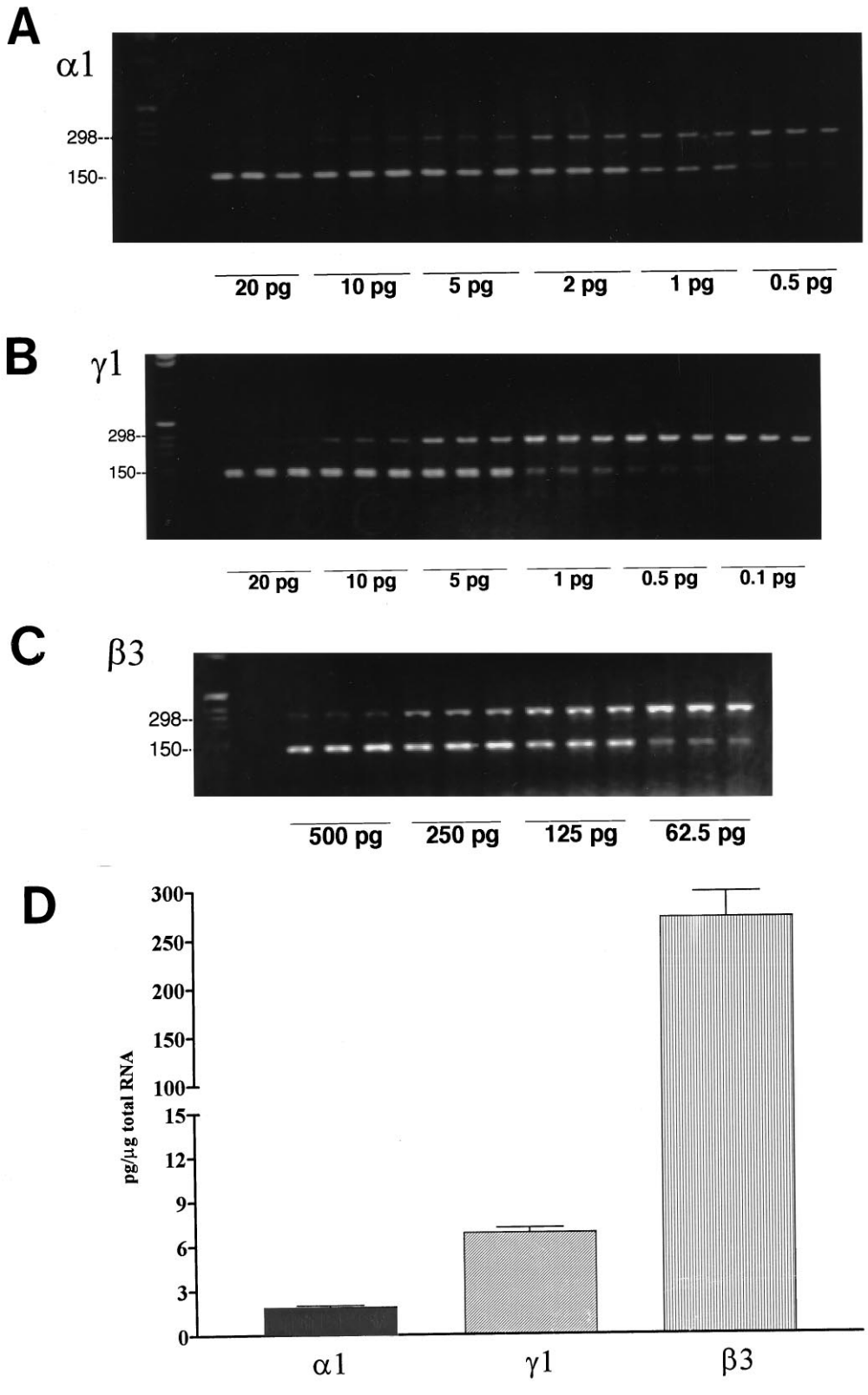

Figure 4. *A–C*, Representative gels for $GABA_A$ receptor subunit mRNAs from cultured E14 rat brainstem analyzed by competitive RT-PCR using internal standards. A series of concentrations of internal standard cRNAs were added to each tube containing 1 μg of total RNA. The PCR products from each tube are shown in triplicate for each subunit. *Top bands*, PCR products of target mRNA. *Bottom bands*, *Bgl*II-digested internal standard PCR products. Note that increasing concentrations of internal standards compete with target mRNA for amplification. The point of equivalence was determined by linear regression analysis of the ratio of counts incorporated into the target PCR product across the series of concentrations of internal standards. The point of equivalence (when the ratio is 1) is the absolute concentration of $GABA_A$ receptor subunit mRNA/microgram of total RNA. *D*, Quantification of $GABA_A$ receptor subunit mRNA levels assayed in this study. Note that β3 is the most abundant subunit compared with α1 and γ1. Cells were cultured for 1 d in DMEM + 10% fetal calf serum and then for 2 d in serum-free medium (DMEM + ITS + 0.1% BSA).

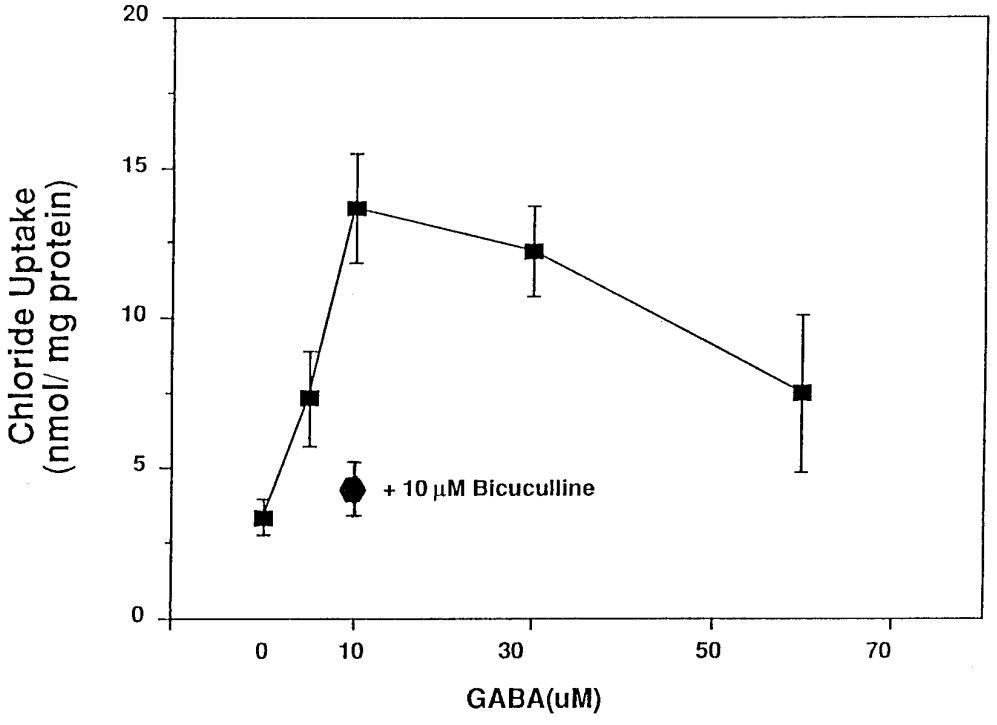

Figure 5. $GABA_A$ receptor-mediated Cl^- uptake into cultured E14 brainstem cells. Addition of exogenous GABA enhanced Cl^- uptake from basal levels of 3.3 nmol/mg protein in a dose-dependent manner to 13.6 nmol/mg protein (at 10 μM GABA). Addition of 10 μM bicuculline lowered Cl^- uptake stimulated by 10 μM GABA to 4.3 nmol/mg protein.

TABLE 1. Effects of GABAA Receptor Ligands on Neuronal Growth

Neurons	Treatment	SA	FA	NN	NTS
5-HT	CONTROL	173.2 (13.9)	2143.3 (174.5)	2.48 (0.09)	1.330 (0.084)
	GABA	194.0 (23.0)	4051.0 (728.4)	3.21 (0.38)	1.547 (0.377)
	BICUCULLINE	156.0 (11.2)	2400.0 (300.0)	2.40 (0.30)	1.300 (0.250)
	DIELDRIN	147.0 (12.9)	826.5 (145.5)	1.90 (0,15)	1.014 (0.014)
	GABA+BICUCULLINE	180.0 (9.0)	3037.0 (450)	3.10 (0.15)	1.280 (0.200)
	GABA+DIELDRIN	162.5 (13.5)	727.0 (147.0)	1.80 (0.20)	1.000 (0.001)
TH	CONTROL	133.4 (9.3)	1059.1 (112.7)	1.56 (0.21)	1.207 (0.047)
	GABA	187.7 (22.5)	1319.1 (229.7)	2.28 (0.19)	1.173 (0.015)
	BICUCULLINE	128.0 (11.0)	413.2 (57.0)	1.30 (0.20)	1.000(0.002)
	DIELDRIN	136.1 (8.6)	1063.4 (63.4)	1.85 (0.15)	1.000 (0.001)
	GABA+BICUCULLINE	142.7 (12.7)	1294.0 (259.0)	2.05 (0.21)	1.090 (0.040)
	GABA+DIELDRIN	143.3 (7.2)	1205.0 (182.0)	1.85 (0.05)	1.106 (0.054)
GABA	CONTROL	106.6 (11.41)	436.9 (60.5)	1.65 (0.30)	1.040 (0.100)
	GABA	131.5 (17.9)	736.2 (165.1)	1.93 (0.12)	1.013 (0.013)
	BICUCULLINE	154.0 (6.4)	1045.0 (204.0)	1.75 (0.16)	1.085 (0.060)
	DIELDRIN	118.2 (25.5)	900.7 (219.2)	2.17 (0.27)	1.000 (0.003)
	GABA+BICUCULLINE	171.0 (8.2)	605.0 (88.0)	1.45 (0.15)	1.000 (0.003)
	GABA+DIELDRIN	120.4 (23.1)	569.5 (39.4)	2.05 (0.35)	1.029 (0.029)

Values are means of 60 neurons (SEM in parentheses) from 3 experiments using cultures from E14 brainstem. 5-HT: 5-HT neurons; TH: tyrosine hydroxylase neurons; SA: soma area; FA: field area; NN: number of neurites; NTS: number of terminal segments.
: significantly different from control at P<.05 (Bonferroni multiple comparison test)

when it was analyzed by the more conservative Bonferroni test. No significant effects on the NN of GABA neurons were seen after any treatment.

NTS: GABA did not significantly affect the *number of terminal neurite segments* of 5-HT and TH neurons, but both bicuculline and dieldrin significantly decreased this parameter in TH neurons, effects that were reversed by GABA. The NTS of GABA neurons were unaffected by any treatment.

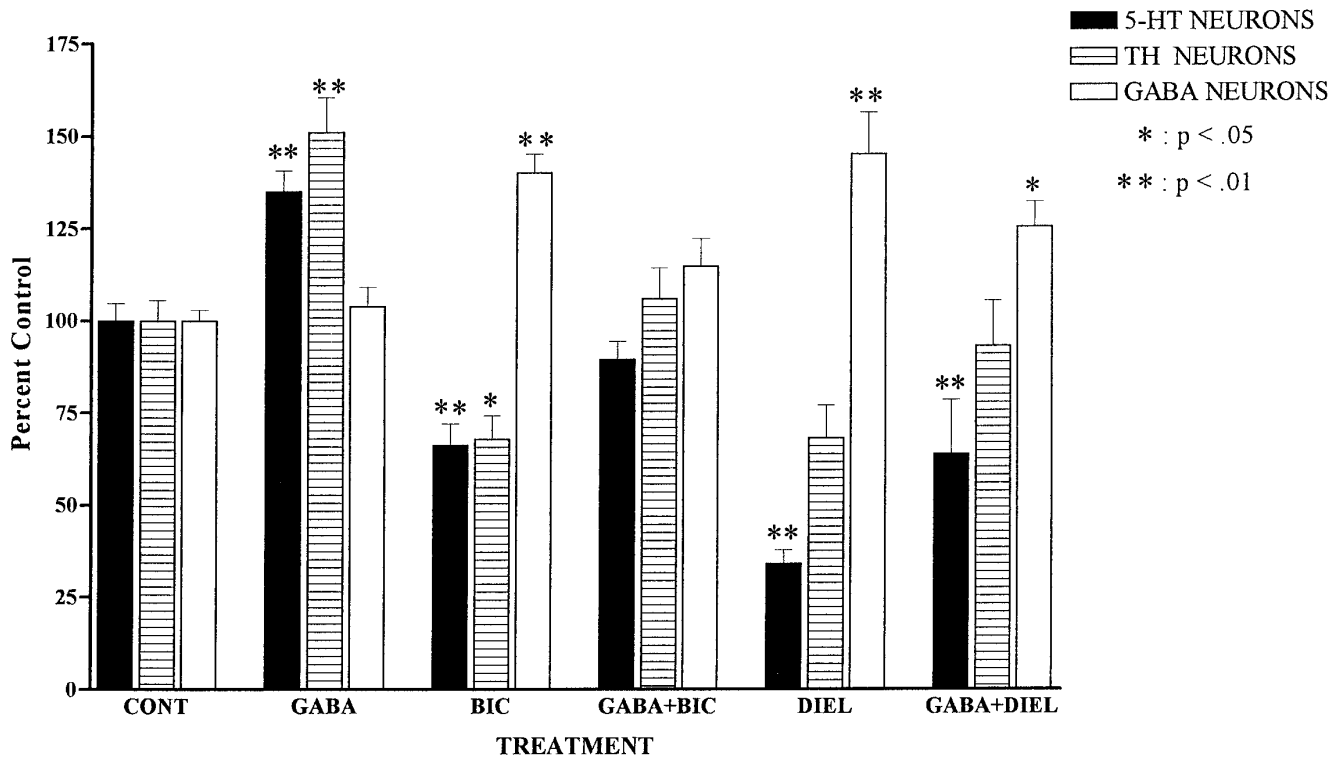

Figure 6. Effects of GABA receptor ligands on survival of 5-HT, TH, and GABA neurons in E14 rat brainstem cultures (number of immunoreactive neurons/mm^2, expressed as percentage control). Cells were cultured for 1 d in DMEM + 10% FCS and then switched to serum-free medium (DMEM + ITS + 0.1% BSA) plus ligand for 48 hr. Cultures were then fixed, stained with antibodies to 5-HT, TH, or GABA, and immunoreactive neurons were counted. Individual data from three separate experiments ($n = 60$ cells/treatment group) were converted to percentage control by dividing individual data points by the overall mean control value. Statistical analysis was performed by ANOVA followed by Dunnet's multiple comparison test when ANOVA was significant ($p < 0.05$).

DISCUSSION

GABA acts as a trophic signal for monoamine neurons but negatively regulates development of GABA neurons

GABA stimulated survival and growth of 5-HT and TH neurons in embryonic brainstem cultures, and these effects were reversed by $GABA_A$ antagonists. These cultures expressed multiple $GABA_A$ receptor subunit mRNA transcripts, as measured by RT-PCR, and exhibited functional GABA-gated Cl^- channels, as determined by $^{36}Cl^-$ influx. The amount of $^{36}Cl^-$ influx stimulated by 10 μM GABA was comparable to values reported previously in whole fetal rat brain (Kellogg and Pleger, 1989), suggesting that GABA-gated channels are highly expressed in these cultures. Taken together, these results suggest that $GABA_A$ receptors mediate the trophic effects of GABA on brainstem monoamine neurons in these cultures. Although it could be argued that GABA-stimulated release of 5-HT from serotonin neurons (Becquet et al., 1993) could account for some of the positive effects of GABA on 5-HT and TH neurons, two points argue against this possibility. First, 5-HT does not promote survival of brainstem monoamine neurons, although it does stimulate their growth (Lauder, 1990; Liu and Lauder, 1991). Second, the $GABA_A$ antagonist bicuculline reversed the positive effects of GABA on monoamine neurons and inhibited growth and survival of these cells when added alone. For these reasons, we believe it is unlikely that released 5-HT played a significant role in the positive effects of GABA on monoamine neurons.

Although GABA itself did not have significant effects on GABA neurons, both bicuculline and dieldrin stimulated survival of these cells, and bicuculline promoted areal growth of neurites (FA). The positive effects of these $GABA_A$ antagonists suggest that GABA may negatively autoregulate development of the GABAergic neuronal population in embryonic brainstem *in vivo*. The lack of significant effects of GABA on GABA neurons in our cultures could be attributable to the presence of endogenous GABA (released by GABA neurons) (Barbin et al., 1993; Becquet et al., 1993), which produced maximal inhibitory effects on these cells before treatment. GABA breaks down within hours after being added to culture medium. Therefore, it is possible that if muscimol had been used instead of GABA, significant negative effects on GABA neurons would have been found; however, because GABA was added daily to the culture medium, and significantly affected growth and survival of monoamine neurons, we think it unlikely that degradation of GABA contributed significantly to the lack of effects on GABA neurons.

Differential effects of receptor ligands suggest heterogeneous expression of $GABA_A$ receptor subunits by monoamine and GABA neurons

The differential effects of $GABA_A$ receptor ligands on cultured monoamine and GABA neurons raise the interesting possibility that these neurotransmitter phenotypes express different amounts of $GABA_A$ receptors and/or distinct $GABA_A$ isoreceptors (subtypes) with differing pharmacological properties. These findings are consistent with evidence that (1) GABA promotes differentiation of different types of neurons to varying degrees in other culture systems

(Hansen et al., 1984; Meier et al., 1985; Spoerri, 1988; Michler, 1990; Belhage et al., 1997) and (2) adult GABA neurons and 5-HT neurons express different complements of particular $GABA_A$ receptor subunits (Gao et al., 1993; Gao and Fritschy, 1994).

Differential effects of $GABA_A$ receptor ligands on growth of cell bodies and neurites (Table 1) raises the further possibility that $GABA_A$ receptors may be differentially distributed on cultured 5-HT, TH, and GABA neurons. This view is supported by previous evidence that (1) high affinity $GABA_A$ receptors are situated on cell bodies of cerebellar granule cells, whereas low affinity $GABA_A$ receptors are preferentially located on cell processes (Hansen et al., 1991); (2) $\alpha 2$, $\alpha 5$, $\beta 3$, and $\gamma 2$ subunit transcripts are expressed by cell somas, neurites, and growth cones of cortical neurons, whereas $\alpha 3$ and $\beta 3$ subunits are confined to cell somas (Poulter et al., 1994); and (3) $GABA_A$ subunit expression on cell bodies and neurites of developing cerebellar granule cells is differentially regulated by the $GABA_A$ agonist THIP (Gaboxadol), suggesting that sorting and targeting of newly synthesized receptors may undergo maturational changes that can be influenced by agonists (Elster et al., 1995).

Organochlorine pesticides affect neuronal growth and survival and alter trophic effects of GABA

Certain pesticides exert their neurotoxic actions by selectively blocking ion channels in the insect nervous system. Organochlorine pesticides such as dieldrin interact with specific sites on the $GABA_A$–Cl^- channel complex to block GABA-induced Cl^- flux in both insect and mammalian cells (Abalis et al., 1986; Gant et al., 1987; Bloomquist, 1992; Pomés et al., 1994a,b). The GABA recognition site has been reported to be targeted by dieldrin (Eldefrawi and Eldefrawi, 1987; Ogata et al., 1988; Tokutomi et al., 1994). Other evidence, however, suggests that dieldrin binds to the picrotoxin site on the Cl^- channel (Nagata and Narahashi, 1994) and suppresses GABA-induced Cl^- currents in a noncompetitive manner (Nagata et al., 1994).

Data from the present study indicate that dieldrin, like bicuculline, has opposite effects on growth and survival of cultured monoamine and GABA neurons. This could be explained by different binding characteristics and sensitivity of these $GABA_A$ receptors to dieldrin. If this is the case, then it would suggest that $GABA_A$ receptors expressed by embryonic monoamine and GABA neurons may have different subunit compositions, because subunit composition is known to affect the binding characteristics and sensitivity of $GABA_A$ receptors to particular receptor ligands (Pritchett et al., 1989; Malherbe et al., 1990; Pritchett and Seeburg, 1990; Burt and Kamatchi, 1991).

Summary

The present study has revealed evidence that GABA acts as a trophic signal for embryonic brainstem monoamine neurons by activating $GABA_A$ receptors, but it may be a negative autoregulatory signal for developing GABA neurons. Consistent with this interpretation, bicuculline and dieldrin had negative effects on monoamine neurons but exerted positive effects on GABA neurons. These differential effects of $GABA_A$ receptor ligands on neurons of serotonergic, noradrenergic, and GABAergic phenotypes raise the possibility that prenatal exposure to pesticides or drugs acting as $GABA_A$ antagonists could interfere with the positive and negative regulatory influences of GABA, thereby producing imbalances in monoaminergic and GABAergic neurotransmission in the developing brain. If long-lasting, these effects could have functional and behavior consequences in offspring. The differential effects of $GABA_A$ receptor ligands on the growth of cell bodies and neurites suggest further that particular cellular compartments may express different levels and/or distinct populations of $GABA_A$ receptor subtypes.

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